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# Composition and variety of the ambrosia fungi associated with ambrosia beetle, *Xylosandrus germanus* (Blandford) (Coleoptera: Scolytidae)

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**Abstract**: The species composition and variety of the fungi in the gallery system and in the mycangium of *Xylosandrus germanus* (Blandford) at various developmental stages were investigated by isolation experiments and scanning electron microscopy. *Ambrosiella hartigii* Batra was the only fungus isolated from the mycangia of the dispersal, boring, overwintering and newly sclerotized adults but not the callow adults. The lowest frequency of the fungus isolated from the mycangium occurred in the newly sclerotized adults. The fungi isolated from galleries consisted of *A. hartigii*, *Acremonium kiliense* Grütz and two species of *Fusarium*. *A. hartigii* occurred in a higher percentage of about 40% – 60% during the period from egg to pupal stage, while *A. kiliense* and *Fusarium* sp.1 accounted for 34.6% and 34.6% from the adult galleries, respectively. *A. hartigii* was always isolated from galleries from the egg to pupal stage. Scanning electron microscopy showed that the conidiophores and conidia of *A. hartigii* were predominantly occurred at the bottom of galleries from the egg to pupal stage, whereas *Fusarium* spp. were predominantly occurred in the mature adult galleries. These results indicated that *A. kiliense*, two species of *Fusarium* as well as *A. hartigii* were associated with *X. germanus* and that the composition and the relative dominance of the fungi in the gallery system and in the mycangium varied with developmental stages of the beetle. This study suggests that the composition and variety of the fungi are likely related to nutritional requirement of larval and adult beetles as well as growth characteristics of the fungi.

**Key words**: *Xylosandrus germanus*; ambrosia fungi; mycangia; gallery; species composition; dominant species

The ambrosia beetle, *Xylosandrus germanus* (Blandford) (= *Xyleborus germanus*, Coleoptera: Scolytidae), originated from Asia (Gauss, 1960; Henin and Versteirt, 2003), has become one of the most important forest pests in Asia, Europe and North America. The beetle has been found in Japan, Korea, the Kuril Islands, Vietnam, Belgium, Switzerland, France and the United States (Nobuchi, 1981; Henin and Versteirt, 2003; Buget and Noblecourt, 2005). It is reported that the beetle has attacked over 120 species of plants, including deciduous and coniferous trees and other woody species. The weakening and dying plants as well as apparently healthy plants have been main targets to be attacked (Weber, 1982; Atkinson *et al*., 1988). Although attacks by *X. germanus* do not always result in tree mortality, tree growth may be seriously reduced (Weber, 1982). Tunnels caused by female beetles considerably reduce timber quality when they bore into trees and excavate a gallery. Economic loss in forest industry has been recorded in European countries (Henin and Versteirt, 2004).

Unlike bark beetles, ambrosia beetles can not utilize phloem and sapwood as food. Instead, both adults and larvae of the beetles feed on fungi. Ambrosia beetles and fungi are mutualistic symbiosis. Ambrosia beetles transport fungi in highly specialized saclike organs termed mycangia and inoculate the fungal spores from the mycangia into the walls of galleries excavated in the wood. The fungi grow in the galleries and become the food of beetles. Of ambrosia fungi, the fungus eaten by larvae and regularly found in mycangia is called as the primary ambroisa fungus (Batra, 1967). Only one primary ambrosia fungus species is associated with a particular beetle species within a particular geographic region (Batra, 1967; Gebhardt *et al*., 2004). Besides the primary ambrosia fungus, other fungal species are found to be associated with the single beetle species. The proportional distribution of the fungal components may vary with the developmental stages of the beetle (Kinuura, 1995).

*Ambrosiella hartigii* Batra (synonym and current name of *Monilia candida* Hartig) has been identified as

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the primary ambrosia fungus associated with *X*. *germanus* in Germany, Japan and the United States of America (Batra, 1967; Weber and McPherson, 1984). Weber and McPherson (1984) reported that *A*. *hartigii* and three species of *Fusarium* were isolated from walls of the beetle's galleries. However, how the proportional distribution and the relative dominance of the fungal components varied with the developmental stages of *X*. *germanus* has been unknown yet.

In China, it was not until the mid 1990s that *X*. *germanus* was reported to infest chestnut (*Castanea mollissima* Blume) (Cheng and Guo, 1994; Wang and Song, 1995). Recently, the beetle began attacking grapevine (Yang *et al*., 2007). The species composition and variety of the ambrosia fungi occurred in grapevine have not yet been known. The objectives of this study are: first, to ascertain the species of ambrosia fungi associated with *X*. *germanus* that attacks grapevine in China; second, to clarify the species composition and the relative dominance of the associated fungi in the gallery system and in the mycangium of *X*. *germanus*. It is expected that the information will be useful in further clarifying the biological characteristics of *X*. *germanus* as well as symbiotic relationship between the beetle and fungi.

## 1 MATERIALS AND METHODS

### 1.1 Field collections of adults and galleries and culture media

The beetles and the infested grape (*Vitis vinifera* L.) stems and branches were obtained from the grapevine orchards (about 700 ha) located in the Nongquanyi District, Chengdu, in southwestern China (515.1 m a.s.l.; 30°33′N, 104°15′E) during 2006 – 2007.

The infested grape branches (about 1 cm in diameter) were cut and transported to the laboratory for obtaining galleries at various developmental stages and the callow and newly sclerotized adult females. The callow and newly sclerotized adults could be discriminated by color of the elytra: the former were white, and the later was light brown.

The galleries were grouped into young/matur larva, pupa, and callow/mature adult galleries by investigating whether the number of the beetles at the same developmental stage was dominant in the gallery. The egg gallery was defined as the gallery in which only the eggs and the mother adult existed.

The dispersal adult females were collected as follows. During the dispersal seasons, the adult females were captured in flight with a small gauze-net and kept separately in vials. The net was rinsed with 95% ethanol and air-dried prior to use in this study. All the adult females were transported to the laboratory for later isolation of fungi from mycangia.

The boring adults were collected when the dispersal adults were boring entrance tunnels, and the overwintering adults were collected when they were overwintering in the grapevine stems in the winter season (from December to next March). The infested grape stems were felled and transported to the laboratory, and then split to expose the adults; and the adults were kept separately in vials for later isolation of fungi from mycangia.

Culture media used in the isolations included potato dextrose agar (PDA), malt extract agar (ME) and yeast extract-malt extract agar (YEME). PDA is a general purpose medium for isolating fungi, and ME and YEME are more specific for isolating ambrosia fungi (Batra, 1967; Roeper *et al*., 1980).

### 1.2 Isolation of fungi from galleries

The infested branches were cut into about 5 cm long logs, and there was only one entrance hole in each log. The surface of each log was rinsed with 95% ethanol and then heated over an open flame. After disinfected three times, the log was split aseptically to expose the brood galleries. The callow adult females and the newly sclerotized adult females in the galleries were placed in sterile vials for later isolation of fungi from mycangia. Samples of the fungal lining were then taken directly from the surfaces of the gallery walls by using sterile forceps, and made into spore suspension with sterile distilled water; the fungal spores were placed on PDA, ME and YEME agar plates by the streak plate method. The cultures were incubated for 3 – 7 days at 25℃ under continuous darkness until colonies were formed, and then purified by further replating.

### 1.3 Isolation of fungi from mycangia

In order to sterilize the body surface, all the adult females were subjected to the following procedures (Francke-Grosmann, 1956): first, the adult females were allowed to crawl on sterile, moist filter-paper in a Petri dish for 2 h; then, they were transferred to another moist Petri dish for 12 h and subsequently incubated on a dry Petri dish for 12 h. This alternating cycle of incubation was repeated three times.

Under a stereo-microscope, each adult sample was transferred to the first drop of water on a glass slide where the prothorax was cut from the head and rest of the body and then transferred to the second drop of water aseptically, and the mycangium was removed by using a sterile needle and placed on PDA, ME and YEME agar plates. The cultures were incubated at 25℃ under continuous darkness, and then the fungi were purified by further replating.

### 1.4　Observation of fungi growing on gallery walls

A few wood blocks（5 mm × 5 mm × 3 mm）containing a piece of the gallery wall were sampled aseptically at each developmental stage of the beetles. The samples placed in vials were fixed with 25% glutaraldehyde fixative for 2 h，dehydrated in a graded ethanol series，dried and mounted on aluminum stubs. Samples were then coated with gold and examined under a scanning electron microscopy（SEM）（KYKY-1000B）operated at an accelerating voltage of 25 kV and a working distance of 12 mm.

### 1.5　Identification of fungi

The fungi were identified according to the morphological and cultural characteristics described by Batra（1967），Booth（1971），and Walter（1971）.

## 2　RESULTS

### 2.1　Fungal flora isolated from galleries of *X. germanus*

Species composition of the associated fungi in the galleries in relation to the developmental stages of the beetle is summarized in Table 1. The fungal species associated with *X. germanus* included *Ambrosiella hartigii* Batra，*Acremonium kiliense* Grütz，and two species of *Fusarium*. The species composition and dominant species varied with the developmental stages of the beetle. Only 2 species of fungi were isolated from the egg galleries，3 from the larval galleries，and 4 from the pupal and adult galleries. From the egg to pupal stages，*A. hartigii* and *Fusarium* sp. 1 occurred in higher frequencies（Table 1）. In the adult galleries，*A. kiliense* and *Fusarium* sp. 1 became dominant，whereas *A. hartigii* occurred in a lower frequency. *Fusarium* sp. 2 always occurred in lower frequencies. *A. kiliense* was not isolated from the egg galleries，and *Fusarium* sp. 2 was not isolated during the egg and larval stages（Table 1）.

**Table 1　Fungi isolated from galleries of *X. germanus***

| Stages of the beetles | Number of tunnels tested（A） | Number of isolates（B） | Ratio of B to A（B/A） | Number of tunnels with the fungus（Percentage*） | | | |
|---|---|---|---|---|---|---|---|
| | | | | *A. hartigii* | *A. kiliense* | *Fusarium* sp. 1 | *Fusarium* sp. 2 |
| Egg | 25 | 43 | 1.72 | 25（58.1%） | 0 | 18（41.9%） | 0 |
| Larva | 23 | 53 | 2.30 | 23（43.4%） | 7（13.2%） | 23（43.4%） | 0 |
| Pupa | 20 | 54 | 2.70 | 20（37.0%） | 14（25.9%） | 15（27.8%） | 5（9.3%） |
| Adult | 18 | 52 | 2.20 | 9（17.3%） | 18（34.6%） | 18（34.6%） | 7（13.5%） |

* Percentage = number of tunnels with the fungus/number of isolates × 100%. The same for Table 2

### 2.　Fungal flora isolated from mycangia of *X. germanus*

The mycangia of the female adults had a simple fungal flora. *A. hartigii* was the only fungal species isolated from the mycangia of the female adults in almost all adult periods but the callow adult period. No fungus was isolated from the mycangium of the callow adult. The frequency of the fungus isolated from the mycangium of the newly sclerotized adult was much lower than that isolated from the mycangia of the dispersal，boring and overwintering beetles（Table 2）.

**Table 2　Fungi isolated from mycangia of *X. germanus***

| Stages of the beetles | Number of mycangia tested（A） | Number of isolates（B） | Ratio of B to A（B/A） | Number of mycangia with the fungus（Percentage） |
|---|---|---|---|---|
| | | | | *A. hartigii* |
| Callow adult | 30 | 0 | 0 | 0 |
| Newly sclerotized adult | 21 | 12 | 0.57 | 12（100%） |
| Dispersal adult | 27 | 25 | 0.93 | 25（100%） |
| Boring adult | 30 | 27 | 0.90 | 27（100%） |
| Overwintering adult | 35 | 33 | 0.91 | 33（100%） |

### 2.3　Fungal morphological characteristics

During the egg and young larvae stages，monilioid conidiophores and a large number of globose conidia were observed growing in the gallery（Fig. 1：A and B）. These characteristic conidiophores and conidia are those of *A. hartigii*. *A. hartigii* formed an about 1.0 mm thick and compact palisade of confluent sporodochia on the walls of galleries. During the period of occurrence of the mature larvae，pupae and callow adults，monilioid conidiophores were observed growing in the gallery，and a few globose spores could be observed（Fig. 1：C，D and E）. However，another species of fungus was found at the bottom of the mature adult galleries（Fig. 1：F）. These conidiophores and conidia are those of *Fusarium* spp. *Fusarium* spp. were predominantly observed in the mature adult galleries.

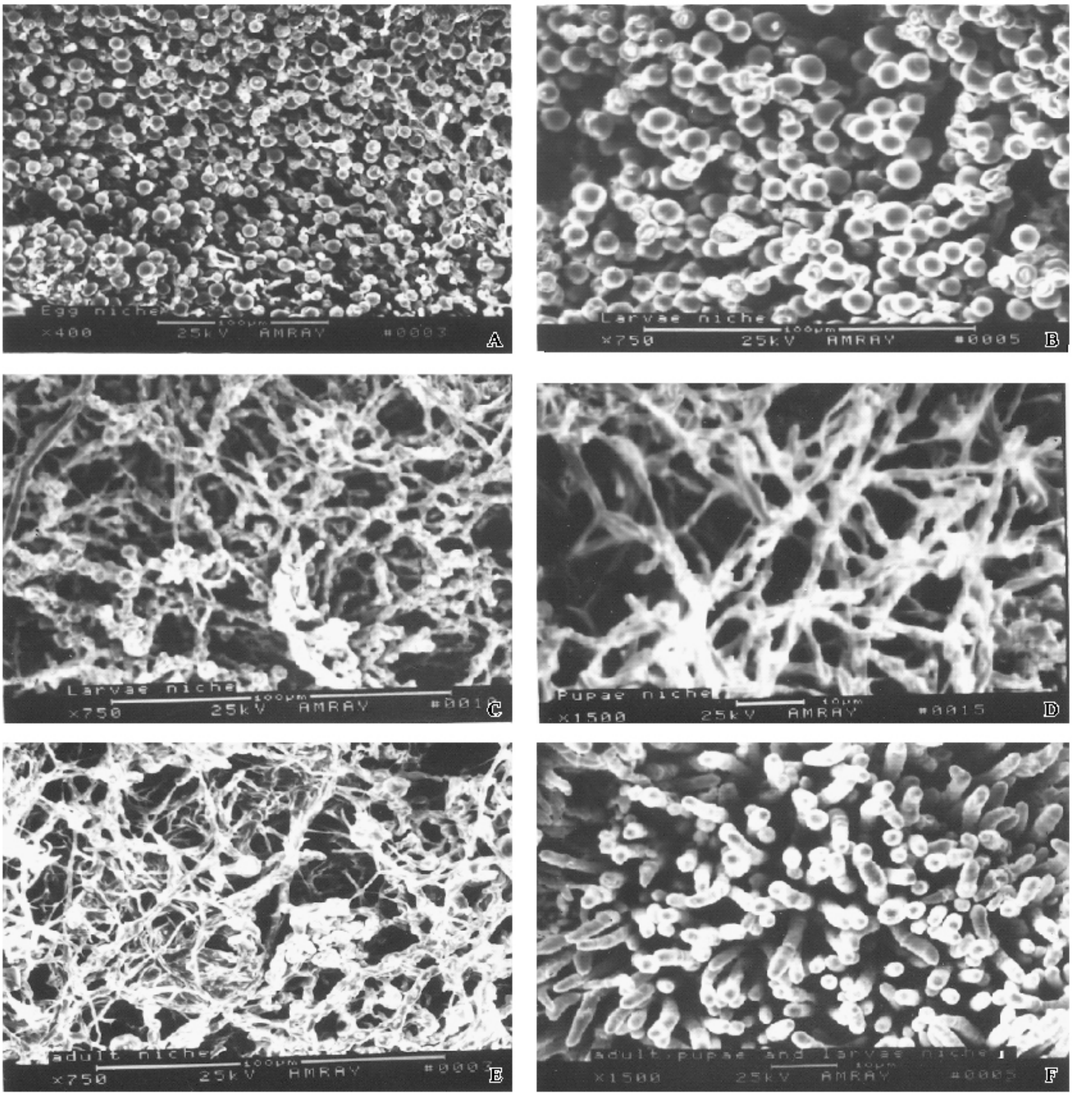

Fig. 1 Fungi microscopic characteristics observed in the gallery at different developmental stages of *X*. *germanus*
A: Egg; B: Young larva; C: Mature larva; D: Pupa; E: Callow adult; F: Mature adult.

## 3 CONCLUSIONS AND DISCUSSION

As expected in the study conducted by Kinuura (1995), in this study, the species composition of the fungi associated with *X*. *germanus* varied with the developmental stages of the beetle. Four fungal species, *i*. *e*. *A*. *hartigii*, *A*. *kiliense* and two species of *Fusarium*, were first isolated from the beetle's galleries in grape stems and branches attacked by *X*. *germanus* in China. This is not in accordance with the results presented by Weber and McPherson (1984) who isolated *A*. *hartigii* and three species of *Fusarium* from the walls of the beetle's galleries.

*A*. *hartigii* was the only fungus found in the mycangium of *X*. *germanus* in this study. This is consistent with previous reports presented by Weber and McPherson (1984). However, our study showed that the frequency of *A*. *hartigii* isolated in the mycangium varied with the development of adult *X*. *germanus*. There was no fungus found in the mycangium of the callow adult but other adults, and the frequency of *A*. *hartigii* isolated from the mycangium of the newly sclerotized adults was much lower than that isolated from the dispersal, boring and overwintering beetles. *A*. *kiliense* was not isolated from the mycangium of adult *X*. *germanus*.

On one hand, the results indicated that existence of *A*. *hartigii* in the mycangium of female adult *X*. *germanus* may be most likely related to competition of *A*. *hartigii* instead of feeding activity. When adults feed on fungi, *A*. *hartigii*, *Fusarium* spp. and *A*.

*kiliense* spores may go into the mycangium by female adults scraping the fungal lining on the walls of galleries when the female adults walked back and forth in the galleries. However，culture on PDA medium showed that *A*. *hartigii* fungus grew much faster than *Fusarium* spp. or *A*. *kiliense*，and *Fusarium* spp. much faster than *A*. *kiliense* in this study. When two fungus species encounter，the growth of *Fusarium* sp. or *A*. *kiliense* was dramatically delayed to a halt. Meanwhile，*A*. *hartigii* continues growing and finally covers the territory of *Fusarium* sp. or *A*. *kiliense*. Takagi and Kaneko suggested that the propagation of *A*. *hartigii* spores in the mycangium of *X*. *germanus* may be occurred during pre-breeding period（Takagi and Kaneko，1965）. Therefore，out-competition of *A*. *hartigii* over *Fusarium* sp. and *A*. *kiliense* results in full occupation of *A*. *hartigii* in the mycangium of *X*. *germanus*. On the other hand，adult females reserved fungus *A*. *hartigii* in their mycangia to facilitate the spread of this fungus and larval survival. While female adults bored into the host plant，*A*. *hartigii* fungus spores were released into the tunnels and started to grow on the wall of galleries. When a great number of conidiophores and spores were formed，the gravid female deposited eggs on the conidiophores and spores to enable newly hatched larvae to get enough food. It has reported that the spores of *A*. *hartigii* were main food resource for the larval development（Batra，1966）. Feeding on the spores of *A*. *hartigii* could accelerate the larval development（personal observation，unpbl.）.

Although four fungus species were found in the gallery system，the proportional distribution and the relative dominance of the associated fungi varied with the developmental stages of the beetle. *A*. *hartigii* was a primary ambrosia fungus among the fungi isolated from attacked grapevine in China. The result is similar to that reported in Germany，Japan and the United States of America（Batra，1967；Weber，1984）. In this study，*A*. *hartigii* was predominant in the galleries over *A*. *kiliense* and *Fusarium* spp. from the egg to pupal stage，and *Fusarium* spp. became predominant over *A*. *hartigii* in the adult galleries. This is most likely related to nutritional requirement of larval and adult *X*. *germanus*. Scanning electron microscopy indicated that the number of *A*. *hartigii* spores obviously decreased with the larval development. A great number of spores existed at the egg and young larva galleries while a few spores were found at the mature larva and adult galleries. After a number of *A*. *hartigii* spores were fed by larvae，*Fusarium* spp. on the wall of galleries might gain enough space and nutrients and grew rapidly. This may account for the reason why *Fusarium* spp. became dominant at the mature adult stage.

It is known that adult *X*. *germanus* feeds on fungi（Weber and McPherson，1984）. However，fungal species remains unclear. *Acremonium kiliense* was first recorded as one of ambrosia fungi associated with *X*. *germanus* in this study. It is reported that *A*. *kiliense*，once considered as a root-colonizing fungus，existed in a dormant state inside the nests of some other insects（Rodmgues *et al*.，2005）. Further evidence showed that it was difficult for adult *X*. *germanus* to lay eggs only with *A*. *hartigii*，although the larvae could complete their development by feeding on *A*. *hartigii*，*Fusarium* spp. and *A*. *kiliense*（personal observation，unpbl.）. *Fusarium* spp. and *A*. *kiliense* may provide some nutrients essential to adult reproduction.

In summary，this study revealed fungal species and components in the gallery and mycangium of *X*. *germanus* and their dynamics associated with developmental stage of this beetle. Four fungal species existed in the galleries，and only *A*. *hartigii* was found in the mycangium. Variation in frequency of the fungi with the developmental stage of *X*. *germanus* was most likely related to nutritional requirements of the larva and adult as well as growth characteristics of the fungi. Further study is needed to understand the symbiontic relationship between fungi and the beetle.

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# 光滑足距小蠹虫道真菌的组成和变化

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**摘要：本文同时采用分离和电镜扫描技术研究了光滑足距小蠹** *Xylosandrus germanus* (Blandford)**不同发育阶段坑道和贮菌器内真菌种类的组成和变化。** *Ambrosiella hartigii* **是光滑足距小蠹扩散、蛀孔、越冬和体壁新硬化的成虫贮菌器内唯一分离到的真菌，而刚羽化的雌成虫贮菌器中没有分离到任何真菌种类，体壁新硬化的成虫贮菌器中真菌的分离频率最低。坑道内共分离到** 4 **种真菌：** *A. hartigii*，*Acremonium kiliense* **和** 2 **种镰刀菌** *Fusarium* spp.**。从卵期到蛹期的坑道中，** *A. hartigii* **总是能被分离到，占分离物总数的百分率较高，达** 40%～60%；**而在成虫期坑道中，** *A. kiliense* **和** *Fusarium* sp.1 **被分离率较高，都为** 34.6%**。扫描电镜观察表明，** *A. hartigii* **为卵期到蛹期坑道中的优势种，而镰刀菌** *Fusarium* spp.**则是成虫期坑道中的优势种。这些结果均表明，** *Ambrosiella hartigii*，*Acremonium kiliense* **和** 2 **种镰刀菌** *Fusarium* spp.**是光滑足距小蠹的虫道真菌，而且这些真菌种类组成和优势种类是随着蠹虫的发育阶段而变化的。真菌种类的组成和变化可能与幼虫和成虫的营养需求以及虫道真菌的生长特性有关。**

**关键词：光滑足距小蠹；虫道真菌；坑道；贮菌器；种类组成；优势种**

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